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HMGCoA reductase Potentiates *hedgehog* Signaling in *Drosophila melanogaster*

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Summary

***Drosophila HMGCoA reductase* (*hmgcr*) catalyzes the biosynthesis of a mevalonate precursor for isoprenoids and has been implicated in the production of a signal by the somatic gonadal precursor cells (SGPs) that attracts migrating germ cells. Here, we show that *hmgcr* functions in the *hedgehog* (*hh*) signaling pathway. When *hmgcr* activity is reduced, high levels of Hh accumulate in *hh*-expressing cells in each parasegment, while the adjacent "Hh-receiving" cells cannot sustain *wg* expression and fail to relocalize the Smoothened (Smo) receptor. Conversely, ectopic Hmgcr upregulates Hh signaling when it is produced in *hh*-expressing cells, but has no effect when produced in the receiving cells. These findings suggest that Hmgcr might orchestrate germ cell migration by promoting the release and/or transport of Hh from the SGPs. Consistent with this model, there are substantial germ cell migration defects in *trans* combinations between *hmgcr* and mutations in different components of the *hh* pathway.**

Introduction

The embryonic gonad of *Drosophila* is generated by the coalescence of two distinct cell types: the somatic gonadal precursors (SGPs) and the germline precursors or pole cells (Boyle and DiNardo, 1995; Boyle et al., 1997; Moore et al., 1998). The SGPs are of mesodermal origin and are formed midway through embryogenesis in three bilateral clusters in parasegments (PS) 10–12. By contrast, the pole cells originate earlier in development at the posterior pole of the precellular blastoderm embryo. When the blastoderm cellularizes, the pole cells are not incorporated into the somatic epithelium and remain on the outside surface of the embryo. In order to reach the mesodermal SGPs, the pole cells (also termed germ cells) must not only be internalized, but they must also migrate through the differentiating tissues of the developing embryo (reviewed in Santos and Lehmann, 2004b). Attractive signals generated by the SGPs are thought to be important in directing migration of germ cells toward the SGPs and subsequently in establishing germline soma cell:cell contacts. The first gene implicated in the production or activity of an attractant by the SGPs was *hmgcr* (*columbus*), which encodes HMGCoA reductase (Van Doren et al., 1998). In *hmgcr*$^{-}$ embryos, the germ cells, instead of migrating toward the SGPs, either remain associated with the basal surface of the mid-gut or scatter in the mesoderm. Conversely, when *hmgcr* is ectopically expressed, the germ cells are induced to migrate toward the cells expressing the Hmgcr protein. Consistent with the idea that *hmgcr* functions in the production or activity of an SGP-specific attractant, Van Doren et al. (1998) found that the expression of *hmgcr* RNAs becomes progressively restricted to the gonadal mesoderm, and the presumptive SGPs, in the period immediately prior to germ cell migration. Interestingly, inhibitor studies have also implicated HMGCoA reductase in the migration of germ cells in Zebra fish; however, unlike in flies, HMGCoA reductase is uniformly expressed in the fish while the germ cells are migrating (Thorpe et al., 2004).

Another gene implicated in the production of an attractant by the SGPs is *hedgehog* (*hh*) (Deshpande et al., 2001; for a review of *hh* signaling, see Lum and Beachy, 2004). Like *hmgcr*, ectopic expression of *hh* induces germ cells to migrate toward the cells, inappropriately producing the Hh signaling protein. Consistent with the idea that the germ cells are responding directly to Hh, we found that several of the cell-autonomous components of the *hh* signaling pathway are required in germ cells for normal migration. Thus, abnormalities in germ cell migration were observed in the progeny of mothers carrying germline clones for mutations in the *hh* pathway genes *smoothened* (*smo*), *fused* (*fu*), *patched* (*ptc*), and *protein kinase A* (*pka*). As would be expected from the known roles of these four genes in the reception of the *hh* signal, the phenotypes produced by *smo* and *fu* germline clones are similar and quite distinct from those observed for *ptc* and *pka*. Moreover, the migration defects observed in *smo*/*fu* and *ptc*/*pka* germline clones can be explained by the role of these genes in the *hh* signaling pathway. *smo* and *fu* are required to respond to the Hh ligand. As might be predicted for cells that can't detect and/or respond to an attractive signal from the SGPs, many of the *smo* or *fu* germ cells scatter through the mesoderm. Conversely, in the absence of maternal *ptc* or *pka*, downstream effectors in the *hh* pathway should be activated to a high level independent of the Hh ligand. As might be predicted if the *hh* response pathway is inappropriately switched on in the absence of ligand, many of the *ptc* or *pka* germ cells clump prematurely and then remain in place instead of migrating toward the SGPs.

Although these findings support the idea that *hh* signaling helps guide germ cells toward the SGPs, there are a number of important questions that remain unanswered. One especially puzzling problem is that there are many sources of Hh in the embryo that could potentially signal to the migrating germ cells. In the ectoderm, Hh is expressed in a stripe pattern in each parasegment (Heemskerk and DiNardo, 1994), while, in the mesoderm, it appears to be expressed not only in the SGP cells, but also in the fat body precursor cells in more anterior parasegments (Deshpande et al., 2001). If Hh protein emanating from these different sources were able to signal the germ cells as they migrate toward the SGPs, the cells should be diverted toward in-

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appropriate somatic targets. Thus, if the *hh* pathway is to function in directing germ cell migration, there must be mechanisms to ensure that the *hh* signal emanating from the SGPs can be specifically recognized by the germ cells. One possibility is that the SGPs produce a second signaling molecule that functions together with Hh to attract germ cells to the SGPs, and prevent them from being directed toward the other extraneous sources of Hh. Another (not necessarily mutually exclusive) possibility is that there are mechanisms that specifically potentiate the activity and/or movement of the Hh protein produced by the SGPs. In considering possible potentiation mechanisms, we noted that Van Doren et al. (1998) found that, while *hmgcr* is broadly expressed in the embryo early in development, it becomes restricted to the SGP cells after the germ cells begin their migration. If *hmgcr* functions to augment the activity and/or movement of the Hh protein, the fact that its expression is limited to the SGPs would provide a mechanism for distinguishing Hh produced by the SGPs from Hh expressed by other cells, such as the fat body precursor cells. In the studies reported here, we show that *hmgcr* functions as a component of the *hh* pathway signaling in several different developmental contexts. Moreover, our data indicate that Hmgcr helps to mediate the release of the Hh ligand from Hh-expressing cells and/or its subsequent movement.

Results

hmgcr Mutations Suppress the Gain-of-Function Wing Phenotypes of $hh^{Moonrat}$

hh is expressed exclusively in the posterior compartment of the wing disc and orchestrates wing development by signaling the expression of downstream target genes such as *decapentaplegic* (*dpp*) and *ptc* in the anterior compartment. In the absence of *hh* signaling, these target genes are not properly activated, resulting in defects in growth and patterning along the anterior/posterior axis. Conversely, when *hh* is inappropriately expressed in the anterior compartment, it activates *dpp* in a pattern that leads to overgrowth of anterior tissues and the partial duplication of distal wing structures (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Tabata et al., 1995). These gain-of-function phenotypes are associated with a dominant *hh* mutation, $hh^{Moonrat}$ (hh^{Mrt}), that causes a partial transformation of anterior wing to posterior (Felsenfeld and Kennison, 1995). The anterior-to-posterior transformations induced by the *Mrt* allele can be dominantly suppressed by mutations in *hh* signaling pathway genes that are required to promote *hh* signaling in either the sending or responding cell. To assess if *hmgcr* influences *hh* signaling in the wing, we tested for genetic interactions with *Mrt*. As positive controls we used mutations in the *hh* signaling pathway gene *dispatched* (*disp*), which is thought to function in the sending cell (Burke et al., 1999).

The *Mrt* wing blades were assigned to five different classes (classes I–V: see Figure S1 in the Supplemental Data available with this article online) based on the severity of the wing phenotype (Felsenfeld and Kennison, 1995). As indicated in the graph in Figure 1, roughly 75% of the control *Mrt* wing blades (hh^{Mrt}/TM3) fall into classes III (Figure 1A) and IV, which represent moderate to relatively severe wing deformations. We found that the phenotypic effects of *Mrt* can be dominantly suppressed by the *hmgcr* mutation, and 75% of the wing blades in $hh^{Mrt}/hmgcr^{1}$ *trans*-heterozygotes belong to either classes I or II (Figures 1B and 1C), which represent nearly normal wing morphology. Moreover, as

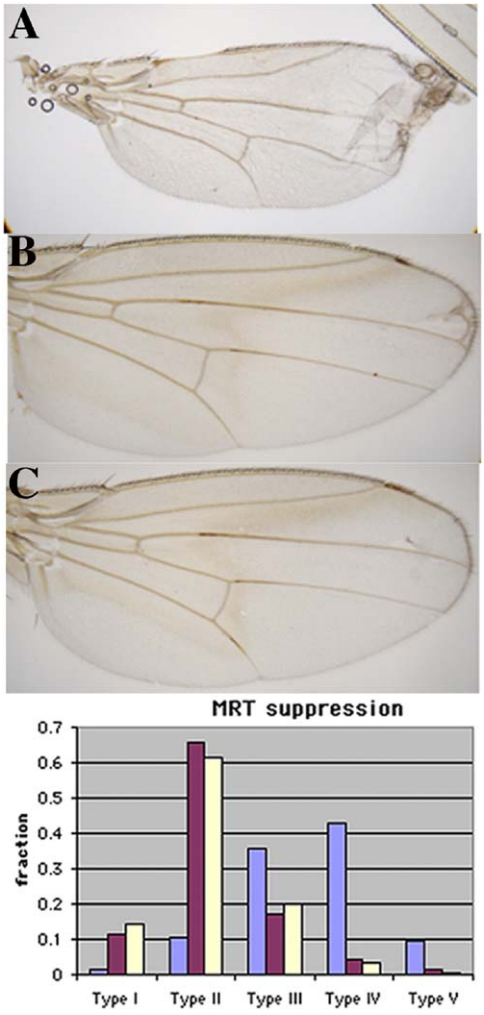

Figure 1. *hmgcr* Can Dominantly Suppress the Wing Abnormalities, Including Pattern Duplication Induced by hh^{MRT}

(A) Ectopic expression of Hh in the anterior compartment of the wing in hh^{MRT}/+ flies induces wing defects, like in the class III wing shown here.

(B and C) Wing defects are suppressed in $hh^{MRT}/hmgcr^{11.57}$ *trans*-heterozygotes. (B) shows a class II wing, while (C) shows a class I wing. The bottom panel shows a graphic representation of the frequency of wing defects in each class seen in different genetic combinations. Blue bars, hh^{MRT}/+; red bars, $hh^{MRT}/hmgcr^{11.57}$; yellow bars, $hh^{MRT}/disp$. For hh^{MRT}/+, 244 single wing blades were analyzed; for $hh^{MRT}/hmgcr^{11.57}$, 243 wing blades were scored; while for $hh^{MRT}/disp$, 229 wing blades were scored. The wings were classified into five different categories depending on the severity of the phenotype by using the criteria described in Felsenfeld and Kennison (1995).

Table 1. Cuticular Phenotypes Induced by *hmgcr*

Phenotypes	Total Number of Embryos (79)
Class I: abnormal cuticle	12 (15%)
Class II: segmental fusions	21 (27%)
Class III: 7-8 fusions	17 (22%)
Class IV: normal segmentation	29 (37%)

Females carrying germline clones for *hmgcr*^{11522} were mated with *hmgcr*$^{11.57}$ males, and the resulting embryos were analyzed. Class I, abnormal cuticle: this class consisted of embryos that typically had no apparent or poorly formed cuticle. If formed, mouth parts and/or filzkorper were formed properly. (This group is likely underestimated because it was difficult to distinguish between unfertilized eggs and embryos that fail to form any discernable structures). Class II, segmental fusions: various segmental fusions and extra denticles. Class III, fusion of abdominal segments 7 and 8. Class IV, normal segmentation: while there were no apparent abnormalities in segmentation, only 12 of the 29 embryos in this class hatched.

shown in the graph, the extent of suppression of the *Mrt* wing phenotypes by *hmgcr* is equivalent to that observed when a *disp* mutation is *trans* to *hh*^{Mrt}.

A Connection between *hmgcr* and the *hh-wg* Regulatory Circuit

One model that could explain the suppression of the *Mrt* wing phenotypes is that *hmgcr* potentiates *hh* signaling. If this is correct, then *hmgcr* mutants might be expected to exhibit segmentation defects similar to those of known *hh* pathway genes (Ingham, 1998; Johnson and Scott, 1998). To explore this possibility, we examined cuticles of *hmgcr* embryos. We found that nearly 30% (14/49) of the *hmgcr* embryos showed fusions of one or more segments and/or the deletion of pattern elements characteristic of mutations in segment polarity genes. The most prevalent defects were the fusion of abdominal segments 7 and 8 (9/14); however, more severe disruptions in patterning were also evident (see Figure S2). The same types and range of patterning defects were observed for another *hmgcr* allele. The frequency of such defects in control embryos is never more than 3%–5%, in our hands.

Although segment polarity defects are clearly evident in *hmgcr*$^{-}$ embryos, the cuticle phenotypes are much less severe than those seen for genes like *hh* and *wg* (which give a lawn of denticles). One explanation for the relatively weak segment polarity defects is that maternally derived Hmgcr compensates for the lack of the zygotic gene product. To test this possibility, we generated *hmgcr* germline clones. While we did not obtain fertile females for the strong *hmgcr*$^{11.57}$ allele, fertile females were obtained for the hypomorphic allele *hmgcr*^{11522}. These females were mated to either *hmgcr*$^{11.57}$*/TM3 Ubx-LacZ* or wild-type males. Table 1 shows a compilation of the cuticle phenotypes observed when the germline clone females were mated to heterozygous *hmgcr* males. The embryos could be divided into roughly four groups (see Table 1). Group I (15%) embryos arrested development without forming cuticle. In a subset of these embryos, abnormal mouth parts and/or filzkorper could be detected (see Figure S2F). Group II (27%) embryos formed at least some cuticle, but embryos had severe segmentation defects. Many of the embryos in this group had deletions/fusions of cuticle pattern elements (see Figures S2C and S2D). In others, cuticle structures like the denticle belts were incompletely formed. Much less pronounced developmental defects were observed in embryos in groups III and IV. Embryos in group III (22% embryos) had fusions of abdominal segments 7 and 8, but were otherwise normal. Embryos in group IV, which represents about 37% of the embryos, resembled wild-type; however, less than half of these animals hatched, suggesting that they may have other vital defects. Since we only observed group III or IV embryos when the *hmgcr* germline clone females were mated to wild-type males (data not shown), we presume that embryos in groups I and II were fertilized by *hmgcr* mutant sperm. Three conclusions can be drawn from these data. First, there is a substantial *hmgcr* maternal contribution. Second, the loss of this maternal product can be partially compensated by zygotic expression from the paternal gene. Third, while *hmgcr* seems to function in the *wg-hh* regulatory circuit, it must have additional roles that are critical for normal development that may be unrelated to the segment polarity pathway.

hmgcr Is Required for Maintaining *wg* and *engrailed* Expression

To provide additional evidence that *hmgcr* functions in segment polarity, we examined the pattern of *wg* expression in *hmgcr* mutant embryos. Up until stage 9/10, we were unable to discern any defects in the pattern or level of *wg* stripe expression in the ectoderm of *hmgcr*$^{z-}$ embryos. However, beginning around stage 11, *wg* expression in *hmgcr*$^{z-}$ embyros is downregulated, and the level of Wg accumulation is reduced compared to wild-type (compare Figures 2A and 2B). Further reductions in Wg protein accumulation are evident in older *hmgcr*$^{z-}$ embryos (data not shown), though even in these older embryos, some residual Wg protein can still be seen in the ectoderm. These findings indicate that *hmgcr* resembles *hh* in that it is not required in the initial activation of *wg* stripe expression in the ectoderm, but is required to sustain *wg* expression. On the other hand, the effects of reduced *hmgcr* activity on *wg* are considerably less severe than those seen in *hh* null mutant embryos. In the absence of *hh*, *wg* stripe expression in the ectoderm disappears almost completely by the end of stage 9, whereas small amounts of Wg protein are still clearly evident in stage 12 and older *hmgcr*$^{z-}$ embryos. This difference could indicate that *hmgcr* activity is not essential for maintaining *wg* expression. Another factor that could contribute to the difference is the substantial maternal contribution of *hmgcr*. To confirm this possibility, we examined Wg expression in progeny from *hmgcr*^{11522} germline clone females mated to *hmgcr* $^{11.57}$/TM3 *Ubx:LacZ* males. As expected, the effects on Wg expression were more pronounced when maternal *hmgcr* activity was compromised (data not shown).

To confirm these findings, we examined the expression of the Engrailed (En) protein in *hmgcr* mutant embryos. *hh* signaling is required to maintain a high level of En expression in the stripes, and, in *hh* mutants, *en*

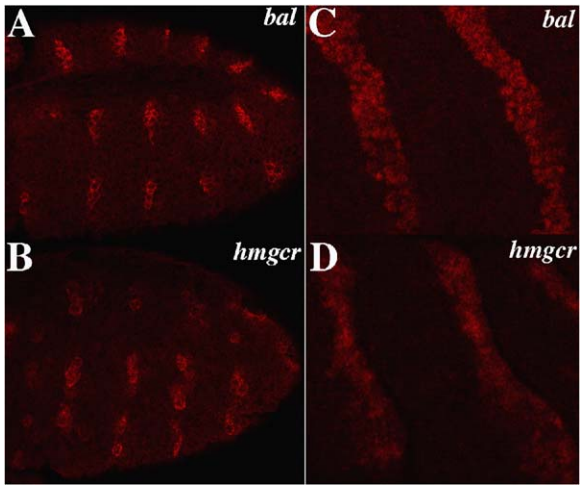

Figure 2. Reduced *wingless* and *engrailed* Expression in Embryos Compromised for *hmgcr*

(A and B) The expression of Wingless protein in a (A) *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* embryo and in a (B) *hmgcr*$^{11.57}$/*hmgcr*$^{11.57}$ embryo. In this experiment, progeny from the *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* stock were stained with β-galactosidase and Wg antibody. *hmgcr*^{z} embryos were identified by the absence of β-galactosidase-specific signal. While stage 9 *hmgcr*$^{z-}$ embryos showed a near wild-type Wg stripe pattern (data not shown), Wg levels started to decline by (B) stage 11 compared to the (A) wild-type. A stronger reduction in Wg protein was seen in embryos that were compromised maternally and zygotically for *hmgcr* function (data not shown).

(C and D) The expression of Engrailed protein in a (C) *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* embryo and in a (D) *hmgcr*$^{11.57}$/*hmgcr*$^{11.57}$ embryo. In this experiment, progeny from the *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* stock were stained with β-galactosidase and En antibody.

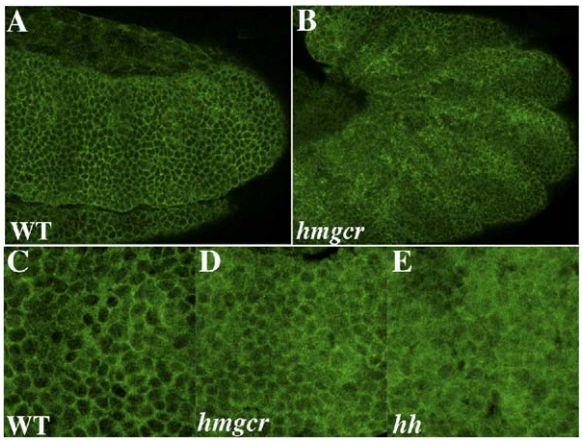

Figure 3. Localization of Smoothened Receptor Is Altered in Embryos Compromised for *hmgcr*

(A–E) Embryos were generated by crossing *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* females with *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* males and were subsequently identified by staining simultaneously with the β-galactosidase and Smo antibodies. (A) and (B) show a stage 10 to stage 11 wild-type *hmgcr*$^{11.57}$/+ embryo and an *hmgcr*$^{11.57}$/*hmgcr*$^{11.57}$ embryo. In wild-type embryos, the Smo protein is segmentally distributed and localizes to the membranes in each segment, whereas a reduced level of signal is seen in the intersegmental region (shown in the magnified view). By contrast, a diffuse and largely cytoplasmic Smo pattern is seen in the *hmgcr* embryos across the parasegment. (C) and (D) show the Smo staining pattern in blow-ups from representative *hmgcr*$^{11.57}$/*TM3; Ubx-LacZ* and *hmgcr*$^{11.57}$/*hmgcr*$^{11.57}$ embryos, while (E) shows the Smo staining pattern in an *hh*/*hh* embryo. Note that the loss of *hh* activity has an even more pronounced effect on Smo relocalization than that observed for *hmgcr*.

expression begins to decay around stages 10–11. As illustrated in Figures 2C and 2D, *hmgcr* is also required to maintain a high level of En expression, and, in embryos lacking zygotic *hmgcr* activity, En expression is reduced compared to wild-type by stage 11.

Relocalization of Smo Protein in *hmgcr* Embryos

The failure to maintain high levels of *wg* expression in older embryos would be consistent with the idea that Hmgcr is required for sending and/or receiving the Hh ligand. To test this hypothesis, we compared the distribution of the Smo protein in wild-type, *hmgcr*$^{z-}$ (*hmgcr*$^{11.57}$), and *hh*$^{-}$ embryos. Previous studies have shown that reception of the Hh signal stabilizes Smo protein and induces it to relocalize from intracellular membrane vesicles to membranes on the cell surface (Denef et al., 2000; Zhu et al., 2003). In wild-type embryos, the effects of Hh signaling on Smo stability and localization can be visualized as a series of stripes that are about five cells wide. In these stripes, Smo is concentrated predominantly at the surface of the cell, giving a ring around the edge of each cell in the stripe in confocal crosssections (Figures 3A and 3C). The stripes are separated by a band of about five cells that have a lower level of localized Smo. As can be seen in Figure 4B, in *hmgcr*$^{z-}$ embryos, the stripe pattern is much less well defined. Moreover, unlike wild-type, the Smo protein is not tightly localized to the cell surface in many of the cells in the stripe, but, instead, it is distributed in the cytoplasm (compare Figures 3C and 3D). Though the Smo localization pattern across each segment in *hmgcr*$^{z-}$ embryos is disrupted, the effects on Smo are not as severe as those seen in *hh* null embryos (compare Figures 3D and 3E).

hh Protein Distribution Is Altered in *hmgcr* Mutant Embryos

The defect in Smo relocalization in *hmgcr*$^{z-}$ embryos supports the idea that Hmgcr activity is required for the production and/or activity of the Hh ligand. To test this possibility further, we compared the pattern of Hh accumulation in wild-type and *hmgcr*$^{z-}$ (*hmgcr*$^{11.57}$) embryos. In wild-type embryos, Hh is expressed in each parasegment in a two cell wide stripe, and the protein in these cells is distributed around the membrane in a punctate pattern (Figure 4A). Extending outward in either direction from the stripe is a relatively sharp gradient of Hh protein. Like the cells in the stripe, the Hh protein associated with the interstripe cells is generally distributed in a punctate pattern around the membrane. No defects in Hh protein expression are apparent in *hmgcr*$^{z-}$ embryos, and, as seen in wild-type, there is a two cell wide stripe of Hh-expressing cells in each parasegment (Figure 4B). Moreover, like wild-type, the protein is concentrated in a punctate pattern around

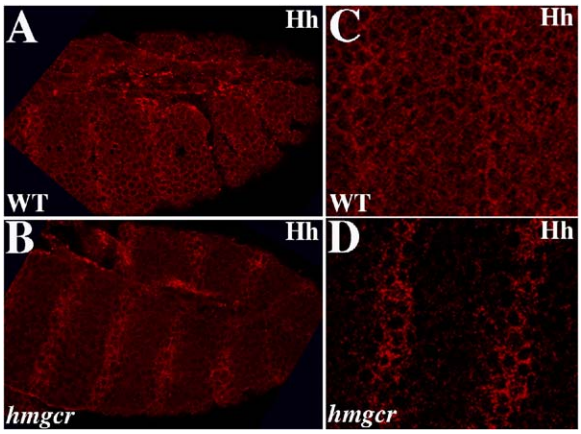

Figure 4. Spread of Hh Ligand Is Restricted in *hmgcr* Embryos

(A and B) Embryos produced by *hmgcr*$^{11.57}$*/TM3; Ubx-LacZ* stock were stained with β-galactosidase and Hh antibodies. Hh-specific signal was imaged with the secondary antibodies coupled to Alexa 546 (red). The figure shows stage 10 to stage 11 embryos of the indicated genotype: (A) wild-type (*hmgcr*$^{11.57}$*/TM3; Ubx-LacZ*; (B) *hmgcr*$^{11.57}$*/hmgcr*$^{11.57}$. In wild-type embryos, two rows of cells per segment express Hh protein. This protein is released from the expressing cells and spreads through the segment.

(C and D) (C) The Hh protein in the interstripe region is clearly visible in the close up of the wild-type embryo. In the *hmgcr*$^{-}$ embryos, Hh accumulates to inappropriately high levels in the stripe of cells that express the ligand (compare [C] with [D]), while the amount of Hh protein in the interstripe region is greatly diminished. This finding suggests that *hmgcr* is required for the efficient release and/or transport of the Hh protein.

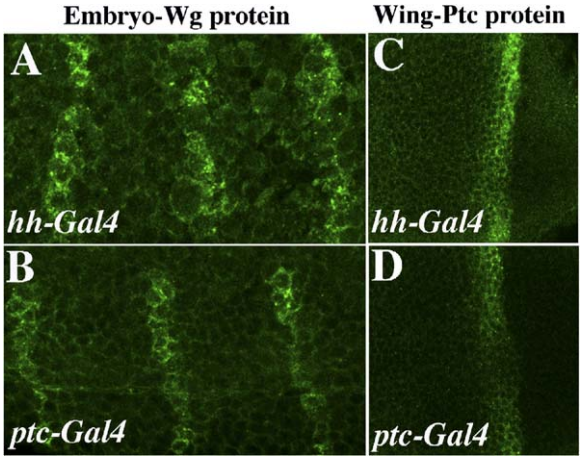

Figure 5. Ectopic Expression of Hmgcr Can Potentiate Hh Signaling in Sending, but Not Receiving, Cells

(A–D) Embryo: (A) and (B) show Wg expression in stage 11 *hh-Gal4* and *ptc-Gal4* embryos, respectively, carrying the *UAS-hmgcr* transgene. Females with two copies of UAS-*hmgcr* were mated with the males of the genotype *hh* GAL4/TM6 *Ubx-LacZ* or *ptc* GAL4/*ptc* GAL4. Embryos (6- to 12-hr-old) were collected, fixed, and coimmunostained with β-galactosidase and Wg antibodies. In the case of the *hh-Gal4* driver, embryos of the correct genotype were identified because there was no β-galactosidase. Staining was visualized by using confocal microscopy, and the staining intensities were compared by using identical settings. As can be seen by the comparison of (A) and (B), *wg* expression is upregulated when Hmgcr is ectopically expressed by using the *hh-Gal4* driver (sending cell), while there is little effect on *wg* when Hmgcr is ectopically expressed by using the *ptc-Gal4* driver (receiving cell). Wing disc: (C) and (D) show Ptc expression in wing discs from third-instar *hh-Gal4* and *ptc-Gal4* larvae, respectively, carrying the UAS-*hmgcr* transgene. Hh in the posterior compartment activates Ptc expression in a stripe along the margin of the anterior compartment. When Hmgcr is ectopically expressed in Hh sending cells by using the *hh-Gal4* driver, it upregulates Ptc accumulation. By contrast, there is little effect on Ptc expression when Hmgcr is ectopically expressed in Hh-receiving cells by using a *ptc-Gal4* driver. The expression pattern of *hh*, *ptc*, and *wg* and the respective *Gal4* drivers can be seen at the bottom of Figure 6.

the cell membrane. On the other hand, the amount of Hh protein in the *hmgcr*$^{z-}$ stripes is considerably higher than wild-type (compare Figures 4C and 4D). Concomitant with the increase in the level of Hh in cells in each stripe, the amount of protein in interstripes is greatly reduced in *hmgcr*$^{z-}$ embryos relative to that seen in wild-type. Similar results were obtained for *hmgcr*$^{m-z-}$.

Activation of *wingless* Expression by Ectopic Hmgcr

The abnormal pattern of accumulation of Hh seen in *hmgcr* mutant embryos suggests that *hmgcr* is required for the efficient release of Hh from the two cells that express this ligand and/or in the transport of Hh from these cells to the adjacent receiving cells. To test this idea further, we examined the effects of ectopically produced Hmgcr on expression of the Hh target gene *wg*. We reasoned that if Hmgcr functions primarily in Hh-producing cells to promote the efficient release or dispersal of the Hh ligand, then overexpression of Hmgcr in these cells might be expected to have a more pronounced effect on *wg* than overexpression in the neighboring Hh-receiving cells. To direct Hmgcr expression in cells that normally produce the Hh ligand, we used an *hh-Gal4* driver, while, for the control, we used either a *ptc* or a *wg* driver to direct Hmgcr expression in cells that normally respond to the Hh ligand.

Figure 5 shows that these expectations are met. In embryos in which Hmgcr is expressed in Hh-receiving cells by using the *ptc* (see Figure 5B) or *wg* driver (data not shown), the pattern of Wg accumulation resembles that of wild-type. Wg is expressed in a single cell wide stripe in each parasegment, and it localizes in these cells in a punctate pattern near the cell membrane. A low level of Wg associated with the membranes of cells in the interstripe region can also be detected. In contrast, when Hmgcr is expressed in Hh-producing cells (Figure 5A), the level of Wg accumulation cells in the *wg* stripe is substantially upregulated. Moreover, we sometimes observe an expansion of the stripe from a single cell to a two cell wide stripe. In addition, high amounts of Wg can be seen extending through much of the interstripe region.

Ectopic Hmgcr Promotes Hh Signaling in the Embryo

The driver-dependent effects of Hmgcr on Wg accumulation would be consistent with the idea that Hmgcr is most effective in enhancing Hh signaling when it is expressed in Hh-producing cells. To test this idea further, we examined the distribution of Hh protein in embryos

in which Hmgcr is expressed under the direction of the *hh*, *wg*, and *ptc* drivers. When Hmgcr expression is directed by the *wg* (data not shown) or *ptc* (Figure 6) drivers, the distribution of Hh protein resembles that seen in wild-type embryos (*bal*). Hh accumulates in a two cell wide stripe in each parasegment, while there is only a relatively low level of Hh protein to either side of this stripe. A different result is obtained with the *hh* driver. Though the Hh parasegmental stripes are still discernable, the stripes are much broader than in wild-type (or when Hmgcr expression is controlled by *wg* or *ptc-Gal4*), and there are high levels of Hh extending to almost the middle of the interstripe region (see blowups in Figure 6). We also examined the effect of ectopic *hmgcr* by using a *paired-Gal4* driver that drives expression in alternate segments. As expected, we found that the breadth of the Hh stripe was only increased in alternate segments (see Figure S3).

Ectopic Hmgcr Promotes Hh Signaling in the Wing Disc

In the embryo, *hh* and *wg* establish an autoregulatory circuit in which signaling by one ligand potentiates signaling by the other. Thus, it is formally possible that the upregulation of *hh* signaling evident when *hmgcr* is overexpressed by using the *hh* driver is the indirect consequence of augmenting the reception of the *wg* signal in *hh*-expressing cells. To exclude this possibility, we tested whether *hh* signaling can be potentiated by ectopic expression of *hmgcr* in *hh*-sending cells in the wing disc, in which there is no autoregulatory circuit between *hh* and *wg*. In the wing disc, the Hh ligand is expressed in the posterior compartment, and it promotes Ptc protein accumulation in the anterior compartment along the compartment boundary. When Hmgcr is expressed in the receiving cells by using the *ptc-Gal4* driver, there is little effect on Ptc accumulation (Figure 5C), and it resembles that in wild-type. By contrast, when Hmgcr is ectopically expressed in *hh*-sending cells by using the *hh-Gal4* driver, Ptc accumulation is upregulated (Figure 5D). These findings indicate that Hmgcr can function in *hh* sending cells in the wing disc to potentiate *hh* signaling.

Genetic Interactions between *hmgcr* and Components of the *hh* Signaling Pathway

Although neither *hh* nor *disp* is haplo-insufficient with respect to germ cell migration, synergistic genetic interactions are observed when mutations in these two genes are combined *in trans*. As illustrated in Figure 7, germ cell migration is essentially indistinguishable from wild-type in embryos heterozygous for an *hh* mutation, and fewer than 20% of the stage 13 to stage 16 embryos have four or more mispositioned germ cells (n = 25). This is also true for embryos heterozygous for a mutation in *disp*. On the other hand, germ cell migration defects are readily apparent in the *trans* combination (Figure 7), and nearly 90% of the stage 13 to stage 16 embryos have ten or more mispositioned germ cells (n = 20).

If the requirement for *hmgcr* function in germ cell migration is related to its role in promoting the transmission or movement of the Hh ligand, then we might

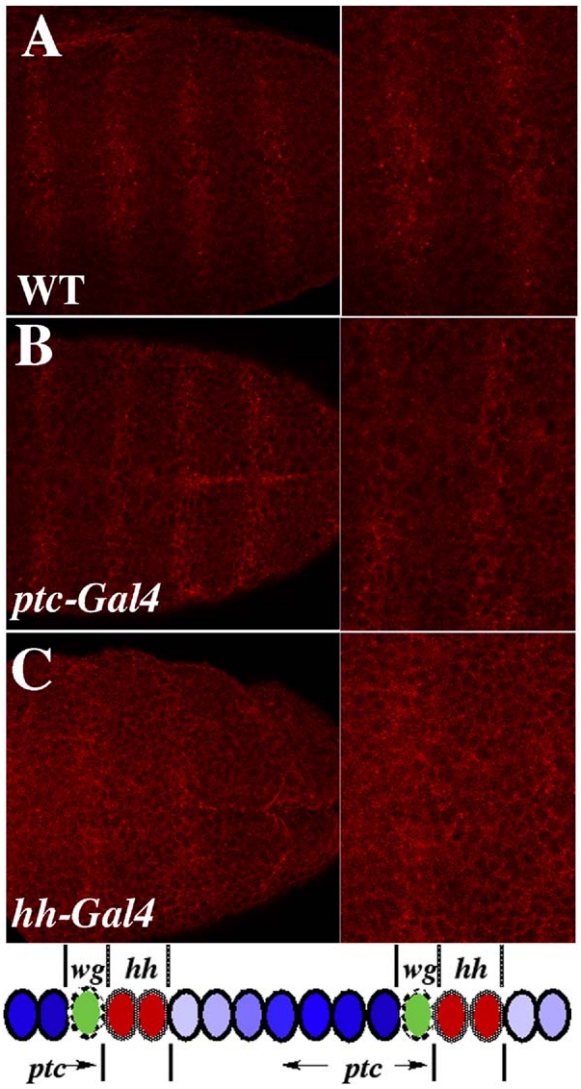

Figure 6. Hmgcr Overexpression in Hh Sending, but Not Receiving, Cells Promotes the Spread of Hh Protein across the Segment

(A–C) (A), (B), and (C) and the accompanying enlargements show Hh expression in stage 11 embryos of the indicated genotypes: (A) wild-type, (B) *UAS-hmgcr; ptc-Gal4*, (C) *UAS-hmgcr; ptc-Gal4* with antibodies against Hedgehog protein. Females carrying two copies of UAS-*hmgcr* were mated with the males of the genotype *hh-Gal4/TM6 Ubx-LacZ* or *ptc GAL4/ptc-Gal4*. Embryos (6- to 12-hr-old) were collected, fixed, and probed with β-galactosidase and Hh antibodies. In the case of the *hh-Gal4* driver, embryos of the correct genotype were identified by the absence of β-galactosidase. The staining was visualized by using confocal microscopy, and staining intensities were compared by using identical settings. As can be seen by the comparison of the bottom panels with the top two panels, Hmgcr is able to promote the spread of Hh ligand only when it is ectopically using the *hh-Gal4* driver (sending cell), but not when overexpressed by using the *ptc-Gal4* driver (receiving cell). Expression patterns of *hh*, *ptc*, and *wg* are represented in the schematic diagram at the bottom. As shown, *hh-Gal4* drives expression in a two cell wide stripe, whereas the *ptc-Gal4* driver is active in a 4–5 cell wide stripe located anterior to the two *hh*-expressing cells. The *wg-Gal4* drives expression in a single cell immediately anterior to the two *hh*-expressing cells. The *ptc-Gal4* driver is also active in this cell.

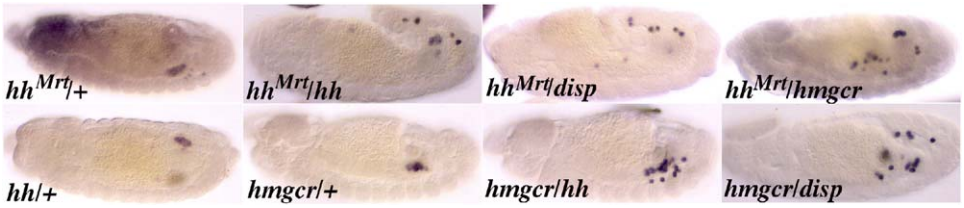

Figure 7. Weak Germ Cell Migration Defects Induced by Perturbing *hh* Signaling Are Enhanced by Reducing *hmgcr*, *disp*, and *hh* Activity

Embryos between stages 12 and 15 of the indicated genotype were stained with anti-Vasa antibody, and staining was visualized with standard immunohistochemcial techniques. The total number of germ cells that failed to associate with SGPs and remained scattered were counted per embryo.

expect to observe equivalent synergistic genetic interactions between *hmgcr* and either *hh* or *disp*. This is the case. There are minor germ cell migration defects in *hmgcr*/+ embryos, and about 35% of the stage 13 to stage 16 embryos have four or more mispositioned germ cells (Figure 7). These minor defects are substantially exacerbated when *hmgcr* is combined with mutations in *hh* or *disp*. In *hh*/*hmgcr trans*-heterozygotes, more than 95% of the stage 13 to stage 16 embryos have ten or more mispositioned germ cells (Figure 7). Similarly, like the *disp*/*hh* combination, a high frequency of germ cell migration defects are evident in the *disp*/*hmgcr trans* combination (Figure 7).

hmgcr Enhances the Germ Cell Migration Defects of hh^{Mrt}

In embryos heterozygous for the *hh* gain-of-function allele hh^{Mrt}, there are defects in germ cell migration (Figure 7), and about 60% of the stage 13 to stage 16 embryos have four or more mispositioned germ cells (n = ~25). Like the wing abnormalities in hh^{Mrt} flies, this weak germ cell migration phenotype is presumed to arise from the misexpression of Hh protein. However, the mechanism is likely to be different from that involved in the mispecification of anterior compartment cells by ectopic Hh. In this case, the ectopic Hh expressed by the *Mrt* allele probably competes with the protein produced by the somatic gonadal precursor cells as an attractant and misdirects the migrating germ cells. If the effects of *Mrt* on migration are due to competition, we reasoned that it should be possible to enhance the germ cell migration phenotype of hh^{Mrt} by reducing the potency of the Hh signal emanating from the somatic gonadal precursor cells. Consistent with this expectation, mutations in both *hh* and *disp* significantly increase the severity of the migration defects seen in hh^{Mrt} (Figure 7), and, in each case, almost all of the embryos (> 90%, n = ~20) had ten or more mispositioned germ cells. As shown in Figure 7, an *hmgcr* mutation also substantially enhances the hh^{Mrt} migration defects, and its effects are equivalent to mutations in either *hh* or *disp*.

Discussion

hmgcr Is a Component of the Hh Signaling Pathway

The pioneering studies of Van Doren et al. (1998) on *hmgcr* indicate that it plays an important role in the synthesis and/or activity of a signal produced by the SGPs to attract germ cells and orchestrate their migration. However, the identity of this signal and how the *hmgcr* might contribute to its synthesis or activity were not established. Additionally, though *hmgcr* is essential for viability, it was not determined whether it functions in patterning and morphogenesis.

In the studies reported here, we show that *hmgcr* functions in the *hh* signaling pathway. This possibility was first suggested by the finding that the wing phenotypes induced by the *hh* gain-of-function allele, hh^{Mrt}, could be dominantly suppressed by an *hmgcr* mutation. Since *disp* mutations also dominantly suppress hh^{Mrt}, this observation indicates that *hmgcr* functions to promote *hh* signaling. Further support for this idea comes from an analysis of the effects of *hmgcr* mutations on *hh* signaling in the embryo. In addition to disruptions in embryonic patterning and *wg* expression characteristic of segment polarity genes, we find that cytoplasmic Smo protein is not properly redistributed to the membrane in *hh*-receiving cells. These defects appear to be due to a failure in the release or transmission of Hh protein from the *hh*-expressing cells; we find that abnormally high levels of Hh accumulate in the membranes of *hh*-expressing cells, while there is a reduction in the amount of Hh protein that is transmitted to neighboring receiving cells.

A role for Hmgcr in the release or transmission of the Hh ligand is also supported by the effects of ectopic Hmgcr. When Hmgcr expression is driven in *hh*-producing cells, it potentiates Hh signaling. First, it upregulates Wg expression in the cell row immediately anterior to the Hh stripe, and it can also weakly induce Wg expression in the neighboring cell row. Second, patterning defects indicative of excessive *hh* activity are evident in newly hatched larvae (data not shown). Third, consistent with a role in releasing Hh from expressing cells or in its transmission to neighboring cells, abnormally high levels of Hh are distributed throughout each parasegment. A quite different result is obtained when Hmgcr is expressed in *hh*-receiving cells by using either a *wg* or *ptc* driver. In this case, there is little or no effect either on Wg expression or on the Hh gradient. Fourth, Hmgcr potentiates *hh* signaling in the wing when it is overexpressed by using a *hh* driver, but it has little effect when overexpressed by using a *ptc* driver. These findings, together with the effects of reducing *hmgcr* function, point to a requirement for Hmgcr activity in *hh*-producing cells, and not in the receiving cells. How-

ever, the precise biochemical function of the Hmgcr protein in this process remains obscure.

In mammals, Hmgcr is required for the synthesis of mevalonate, a precursor for isoprenoids and cholesterol. Since Hh has a cholesterol modification at the C terminus (Porter et al., 1996a, 1996b), one idea is that *hmgcr* functions in this modification. However, Santos and Lehmann (2004a) have made a convincing case that the conventional cholesterol biosynthetic pathway does not exist in flies and consequently that *hmgcr* is unlikely to be involved in cholesterol synthesis. Additionally, the phenotypic effects of Hmgcr misexpression in Hh-producing cells do not seem to be entirely consistent with a function in generating cholesterol-modified Hh. Though somewhat controversial, studies in flies indicate that the cholesterol modification provides a tether for Hh that helps to anchor it to membranes and restrict its range of signaling (Lee et al., 1994; Strigini and Cohen, 1997). By contrast, the expression of excess Hmgcr in Hh-producing cells seems to facilitate the release of Hh protein. In fact, the gain-of-function effects of ectopic Hmgcr on *hh* signaling in the embryo closely resemble those observed when a Hh protein, Hh-N, which is not subject to cholesterol modification, is produced in Hh-expressing cells. Taken together, these observations argue that Hmgcr does not promote *hh* signaling in flies by providing the necessary precursors for cholesterol modification.

Instead, the Hmgcr protein would seem to function either directly in the transport/release of Hh or indirectly through the modification of some factor that is responsible for this process. With respect to the former possibility, it is interesting that, like Disp, Hmgcr is predicted to be a seven-pass transmembrane protein containing a sterol-sensing domain. In Disp, this domain is thought to mediate interactions with cholesterol-modified Hh. Conceivably, the sterol-sensing domain in Hmgcr could perform a similar function. In this case, Hmgcr could interact directly with Hh and function at some step leading up to its release from the sending cell. Consistent with the latter possibility, the biosynthetic product of Hmgcr, mevalonate, is a precursor, not only of cholesterol, but also for a variety of isoprenoids that are used in the modification of proteins. Thus, it is possible that Hmgcr functions in Hh signaling indirectly by synthesizing precursors for a lipid(s) that is used to modify a protein(s) that actually facilitates the release and/or cell-to-cell transfer of the Hh ligand. A function in the synthesis of lipid molecules for protein modifications (or membrane biogenesis/function) is consistent with the finding that the cuticle defects in embryos lacking both maternal and zygotic Hmgcr are much more severe than those seen for mutations in typical *hh* pathway genes. These cuticular phenotypes, together with our failure to obtain germline clones with a strong *hmgcr* allele, argue that besides *hh* signaling, *hmgcr* activity is required for other vital processes. This would be consistent with the finding that mutations in two genes, downstream of *hmgcr* in protein prenylation, farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase, also cause germ cell migration defects (Santos and Lehmann, 2004a).

Function of *hmgcr* in Germ Cell Migration

An important question is whether the role of *hmgcr* in germ cell migration is related to its function in facilitating the release or transmission of the Hh ligand or to its activity in another pathway or process. Though not entirely conclusive, our data on this question point to a function in *hh* signaling. We have found that migration of germ cells toward the SGPs and their subsequent coalescence into the embryonic gonad are very sensitive to the activity of the *hh* signaling pathway, and there are substantial migration defects in embryos *trans*-heterozygous for mutations in *hh* and *disp*. This synergistic genetic interaction can be explained by a weakening of the Hh attractive signal produced by the SGPs in *trans*-heterozygous animals. Consistent with the idea that the function of *hmgcr* in the production of a migration signal from the SGPs is to promote the release or transmission of the Hh ligand, similar synergistic genetic interactions are observed between mutations in *hmgcr* and either *hh* or *disp*. A second line of evidence for a function in the *hh* signaling pathway comes from genetic interactions with the gain-of-function hh^{Mrt} allele. Embryos heterozygous for hh^{Mrt} exhibit minor but consistent defects in germ cell migration. As in wing discs, these defects presumably arise because Hh protein is ectopically expressed by the *Mrt* allele. In this case, however, the ectopic protein competes with Hh emanating from the SGPs in attracting the migrating germ cells. As predicted by this model, weakening the Hh signal from the SGPs exacerbates the effects of the *Mrt* allele, and there are quite pronounced germ cell migration defects in animals *trans*-heterozygous for hh^{Mrt} and loss-of-function mutations in either *hh* or *disp*. Like these two *hh* signaling genes, an *hmgcr* mutation also substantially enhances the *Mrt* migration defects.

These genetic interactions, together with the data of Van Doren et al. (1998), would be consistent with a model that postulates that the function of *hmgcr* in germ cell migration is to facilitate the release and/or transmission of the Hh ligand specifically produced by the SGPs. Van Doren et al. (1998) have shown that *hmgcr* mRNA is initially expressed broadly in the embryo, but, as development proceeds, its distribution becomes progressively restricted, and by stages 11–12 the mRNA is limited to the SGPs in parasegments 10–12. If the pattern of accumulation of the Hmgcr protein mimics that of the message, then the SGPs should be the only cells in the embryo that have high levels of Hmgcr at the time that the germ cells begin their migration from the mid-gut to the gonad. We have shown that when Hmgcr is ectopically expressed in *hh*-producing cells in the ectoderm, it facilitates the release/transmission of the Hh ligand and promotes its spread though the parasegment. It would be reasonable to suppose that Hmgcr acts in a similar fashion on the Hh protein expressed by the SGPs, enabling the SGP-Hh to signal to migrating germ cells over a considerable distance. Since the other Hh-producing cells in the mesoderm, such as the fat body precursor cells in parasegment 9 and more anterior parasegments, do not express *hmgcr* at this point in development, the range or activity of the Hh protein expressed by these cells would be much restricted compared to the SGP-Hh.

Finally, a specific potentiation of the Hh ligand for long-distance signaling by Hmgcr would also explain why germ cells are attracted to cells in which Hmgcr is ectopically expressed (Van Doren et al., 1998).

Experimental Procedures

Strains and Culturing

Flies were grown on a standard medium at 25°C unless otherwise noted. Suppression analysis involving $hh^{Moonrat}$ (hh^{Mrt}) was carried out at 18°C.

Immunohistochemistry

The embryo stainings were performed essentially as described in Deshpande et al. (1995). Anti-Wingless, Anti-Clift, and Anti-Engrailed are all mouse monoclonal antibodies (1:20). Rabbit polyclonal antibodies against Vasa and β-galactosidase (Kappel) were used at 1:500 dilution. Anti-Hh (1:1000; rabbit) and Smo antibodies (1:500; rat) were kindly provided by Tom Kornberg and Steve Cohen, respectively, and were used after preabsorption.

Misexpression Analysis

The following *UAS Gal-4* stocks were used for the misexpression studies: *UAS-hh*, *UAS-disp*, *UAS-hmgcr*, *hairy Gal4*, *patched Gal-4*, *hh-Gal4*.

Typically, males carrying the *UAS* transgenes were mated with virgin females from the *Gal4* stocks (Brand and Perrimon, 1993). The resulting progeny embryos were fixed and stained for subsequent analysis (for details, see figure legends).

Germ Cell Migration Defects

In order to assess the extent of the germ cell migration defects in different mutant backgrounds, including the *trans*-heterozygous combinations, 20 embryos between stages 13 and 15 of each genotype were analyzed, and, in each case, the total number of germ cells that were found to be scattered away from the coalesced gonad were counted.

Germline Clonal Analysis

Germline clonal analysis was performed as described in Chou and Perrimon (1992) by using $FRT\text{-}hmgc^{r11522}$ (Perrimon et al., 1996) and $FRT\text{-}hmgcr^{26.31}$. Females carrying germline clones were mated with the males of the appropriate genotype. Embryos derived from these crosses were fixed and stained with the relevant antibodies for further analysis.

Supplemental Data

Supplemental Data supporting the claim that *hh* signaling is influenced by *hmgcr* are available at http://www.developmentalcell.com/cgi/content/full/9/5/629/DC1/.

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